

## Keratin 20 Expressed in the Endocrine and Exocrine Cells of the Rabbit Duodenum

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The expression of intermediate filaments is sensitively reflected in cell function. To examine the involvement of keratin in a secretory function, 15 kinds of keratin (keratin-2, 3, 4, 5, 6, 7, 8, 10, 13, 14, 16, 17, 18, 19, 20) were detected immunohistochemically and immunoelectron microscopically in the rabbit duodenum. Four types of secretory cells existed in the rabbit duodenum: enteroendocrine cells and goblet cells in the epithelium and mucous and serous cells in the duodenal glands. Among the 15 kinds of keratin, keratin 20 was selectively expressed in all these secretory cells. However, localization of keratin 20 in the endocrine cells differed from that in three types of exocrine cells. In the enteroendocrine cells, keratin 20-containing filaments formed a juxtannuclear network from which they extended to the apical cell membrane. These filaments may play a role in intracellular signal transduction, since the apical cell membrane contains some receptors for binding a specific extracellular signal. In the exocrine cells, on the other hand, keratin 20-containing filaments existed just beneath the cell membrane. These filaments may play some role in maintaining cell shape, which is remarkably changed during the secretory cycle.

**Key words:** keratin 20, intermediate filaments, secretory cell, endocrine cell, small intestine

### I. Introduction

Intermediate filament proteins are highly diverse, and their expression is sensitively reflected in the cell differentiation occurring in histogenesis and disease [8, 15–17, 26]. The intermediate filaments expressed in epithelial cells are mainly keratin (K) filaments which consist of a specific combination of type I keratins (K9–K20) and type II keratins (K1–K8) [1, 14]. However, in rabbits, the M cells of the Peyer's patches, villus epithelium of the small intestine, appendix and palatine tonsil selectively expressed vimentin intermediate filaments [9, 10, 18, 20, 21]. Although the structure of these M cells varies greatly, vimentin filaments were located from the perinuclear region to the cell membrane in contact with the intraepithelial lymphocytes in these cells. Therefore, the kind of intermediate filaments is considered to have a close relationship to cell function rather than cell structure.

In the small intestinal epithelium including the villi and crypt, six types of cells can be distinguished; namely, absorptive cells, goblet cells, endocrine cells, Paneth cells, tuft cells, which are also called brush cells [11, 44], and M cells [18], which were previously called 'cup cells' because of their cell shape [33]. In addition, the tela submucosa of the duodenum contains many duodenal glands of Brunner, which are derived from the intestinal epithelium. Therefore, duodenal epithelial tissue provides a valuable model for the study of the relationship between intermediate filaments and cell function.

The multipotent stem cells of the intestinal epithelium exist in the crypt [22], and produce two kinds of precursor cells as the first step of their differentiation: Math1 gene-expressing secretory precursor cells and non-secretory precursor cells [30, 45]. We previously clarified the relationship between intermediate filament networks and cell function in the non-secretory cells of the rabbit duodenum [18, 19]. This study focuses on the relationship in the secretory cells. The secretory cell population of the rabbit duodenum differs from that of the human duodenum in the absence of Paneth cells in the crypts [46] and in the presence of serous cells in the duodenal glands [25, 28]. Therefore,

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four types of secretory cells exist in the rabbit duodenum; namely, goblet cells and endocrine cells in the epithelium, and mucous and serous cells in the duodenal glands. To examine the keratin intermediate filaments participating in secretory function, 15 kinds of keratin were detected immunohistochemically in the rabbit duodenum.

## II. Materials and Methods

### Immunohistochemical staining

Adult female rabbits weighing approximately 3 kg (Japan white rabbit, Japan Lamb Ltd., Hiroshima, Japan) were anesthetized with an intravenous injection of sodium pentobarbital [0.5 ml/kg]. The duodena were fixed in 4% paraformaldehyde in 100 mM phosphate buffer (pH 7.4) for 4 hr at 4°C and washed in chilled phosphate-buffered saline (PBS) for 16 hr, after which they were dehydrated and embedded in paraffin.

Keratins were detected using the avidin-biotin complex (ABC) method (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA). Deparaffinized sections, 3 µm in thickness, were treated with 0.3% hydrogen peroxide in methanol and immersed in normal horse serum in PBS for 20 min. Then the sections were exposed to the 16 kinds of monoclonal antibodies shown in Table 1 for 1 hr. The antibodies were diluted with PBS containing 0.1% bovine serum albumin (BSA). Before incubation in anti-keratin 4, 8, 10 and 20 antibodies, the sections were pretreated in 10 U/ml trypsin (EC 3.4.21.4, type 1, Sigma) in PBS. After washing in PBS, they were exposed to biotinylated horse anti-mouse

IgG antibodies for 30 min, and then immersed in ABC reagent for 30 min. For visualization of the immunoreactions, they were incubated in a peroxidase substrate solution containing 0.02% 3-3'-diaminobenzidine, 0.005% hydrogen peroxide and 50 mM Tris-HCl buffer (pH 7.6). After immunostaining, they were counterstained with hematoxylin. Negative controls were exposed to PBS containing 0.1% bovine serum albumin or preimmune horse serum instead of the primary antibody. No immunostaining was observed in the negative control sections.

### Immunoelectron microscopical staining

Keratin 20 (K20) was visualized essentially using the ABC method described above except for minor modifications. The duodena were fixed in 4% paraformaldehyde containing 0.1% glutaraldehyde and 100 mM cacodylate buffer (pH 7.4) for 2 hr at 4°C and washed in chilled PBS for 16 hr. Then they were sliced into leaf-like pieces, 30 µm in thickness, using a microslicer (DTK-3000, Dosaka EM, Kyoto, Japan). The sliced tissue samples were incubated in PBS containing 0.3% Triton X-100 and 1% BSA for 2 days at 4°C, and then immersed in 0.3% hydrogen peroxide in methanol for 30 min. After incubation in normal horse serum in PBS for 4 hr, the tissues were exposed to anti-K20 monoclonal antibody solution containing 0.1% BSA and 0.1% Triton X-100 for 3 days at 4°C and were successively washed in PBS. Then they were immersed in biotinylated horse anti-mouse IgG antibodies for 16 hr at 4°C, washed in PBS, and reacted with ABC reagent for 1 hr at room temperature. After washing in PBS, they were

**Table 1.** Monoclonal antibodies used in this study

Antibody	Clone	Supplier	Dilution
anti-cytokeratin 2e antibody	Ks2.342.7.1	Progen* <sup>1</sup>	1:200
anti-cytokeratin 3 antibody	AE5	Cymbus* <sup>2</sup>	1:50
anti-cytokeratin 4 antibody	6B10	Sigma* <sup>3</sup>	1:300
anti-cytokeratin 5 antibody	C-50	Monosan* <sup>4</sup>	1:10
anti-cytokeratin 6 antibody	Ks6.KA12	Progen	1:1
anti-cytokeratin 7 antibody	LDS-68	Sigma	1:200
anti-cytokeratin 8 antibody	M20	Sigma	1:200
anti-cytokeratin 10 antibody	DE-K10	Progen	1:10
anti-cytokeratin 13 antibody	KS-1A3	Sigma	1:100
anti-cytokeratin 14 antibody	CKB1	Sigma	1:200
anti-cytokeratin 16 antibody	LL025	Neo Markers* <sup>5</sup>	1:100
anti-cytokeratin 17 antibody	Ks17.E3	Progen	1:10
anti-cytokeratin 18 antibody	CY-90	Sigma	1:800
anti-cytokeratin 19 antibody	A53-B/A2	Sigma	1:50
anti-cytokeratin 19 antibody	RCK108	Progen	1:20
anti-cytokeratin 20 antibody	Ks20.8	Dako* <sup>6</sup>	1:50

\*1: Progen Biotechnik GmBH, Heidelberg, Germany.

\*2: Cymbus Biotechnology Ltd., Eastleigh, UK.

\*3: Sigma, St. Louis, MO, USA.

\*4: Monosan, AM Uden, The Netherlands.

\*5: Neo Markers, Union City, CA, USA.

\*6: Dako Japan, Kyoto, Japan.

**Table 2.** Keratins expressed in the four kinds of secretory cells

Keratin	Goblet cells	Endocrine cells	Serous cells	Mucous cells
K2e	—	—* <sup>2</sup>	—	—
K3	—	—* <sup>2</sup>	—	—
K4	—	—* <sup>2</sup>	—	—
K5	—	—	+	—
K6	—	—* <sup>2</sup>	—	—
K7	—	—	—	—
K8	—	—	+	—
K10	—	—* <sup>2</sup>	—	—
K13	—	—	—	+
K14	—	—	+	—
K16	—	—* <sup>2</sup>	—	—
K17	—	—	+	—
K18	—	—	+	—
K19* <sup>1</sup>	—	—* <sup>2</sup>	—	—
K20	+	+* <sup>3</sup>	+	+

+ : detected, — : undetected.

\*1: No immunoreaction was recognized by two anti-cytokeratin 19 antibodies shown in Table 1.

\*2: A negative reaction was decided upon because of the absence of positive cells in the epithelium.

\*3: A positive reaction was confirmed immunoelectron microscopically.

incubated in peroxidase substrate solution for 15 min and washed in PBS. Then they were postfixed in 2% osmium tetroxide in 100 mM cacodylate buffer, (pH 7.4) for 60 min at 4°C and for 30 min at room temperature, dehydrated, and embedded in Epon 812. The ultrathin sections were stained with uranyl acetate. Negative controls were exposed to PBS containing 0.1% BSA or preimmune horse serum instead of the primary antibody. No reaction was recognized in the negative control tissues.

For the study of normal ultrastructure, some tissues were fixed in 2.5% glutaraldehyde in 70 mM cacodylate buffer (pH 7.4) for 2 hr and postfixed with 1% osmium tetroxide in 150 mM cacodylate buffer (pH 7.4). After dehydration, they were embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate, and observed with a Hitachi H-7100 electron microscope.

This study was approved by the Animal Research Committee of Kawasaki Medical School (No. 06-045) and conducted according to the "Guide for the Care and Use of Laboratory Animals" of Kawasaki Medical School.

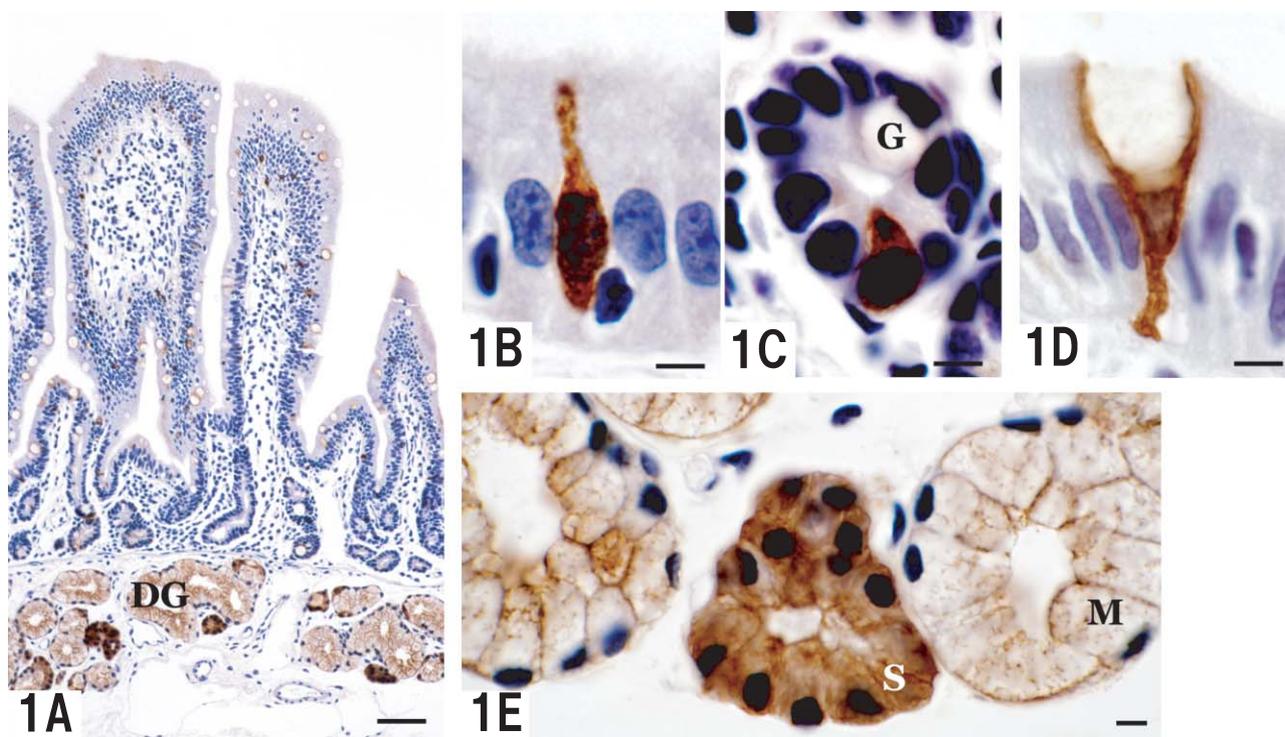
### III. Results

Ultrastructural examination confirmed that Paneth cells were absent in the crypt and that serous cells existed in the duodenal glands. Therefore, four types of secretory cells ex-

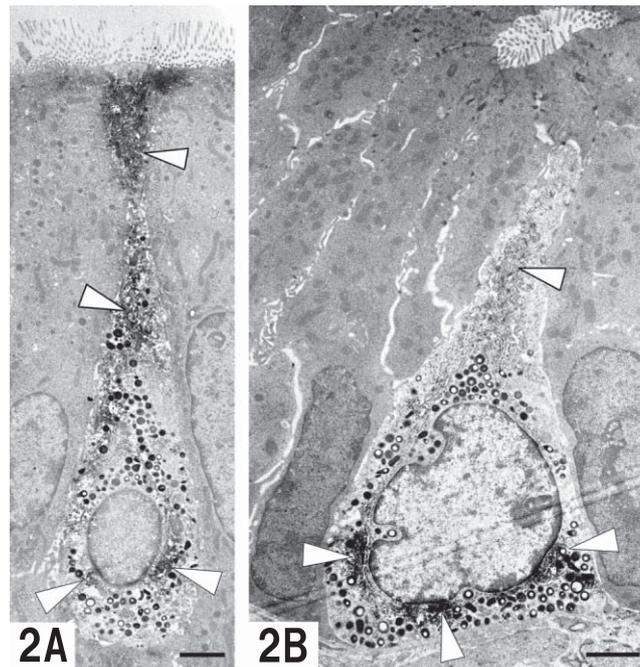
isted in the rabbit duodenum; namely, goblet cells and endocrine cells in the epithelium, and mucous cells and serous cells in the duodenal glands. When rabbit duodena were stained immunohistochemically with the 16 kinds of anti-keratin monoclonal antibodies shown in Table 1, K20 was selectively expressed in four types of cells; namely, in a few special columnar cells scattered throughout the epithelium, and in goblet cells, mucous cells and serous cells (Table 2, Fig. 1). Immature and mature absorptive cells did not express K20, although a small amount of K20 was detected in the absorptive cells just before exfoliation at the tip of villi.

#### *Localization of K20 in endocrine cells*

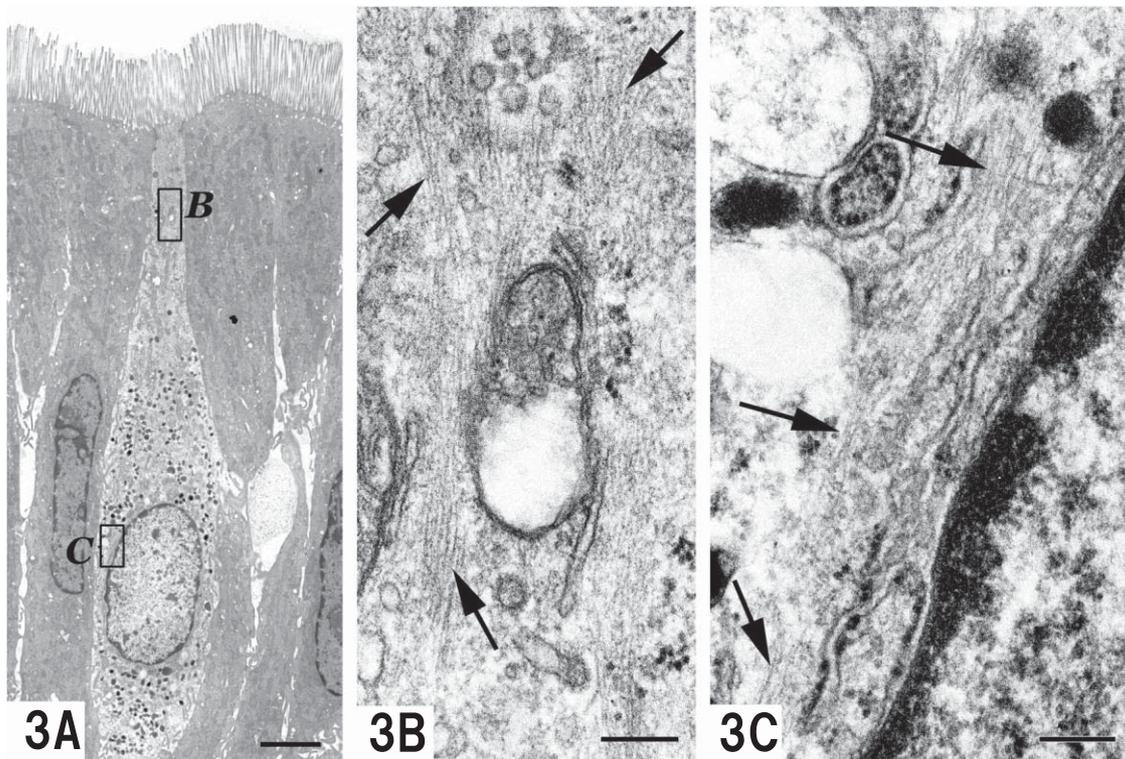
A few special columnar epithelial cells, which abundantly expressed K20, were scattered throughout the epithelia of the villi and crypts (Fig. 1B, C). To clarify the nature of the K20-positive columnar cells, K20 was detected immunoelectron microscopically. All of these columnar cells accumulated many membrane-limited secretory granules in their basal cytoplasm (Fig. 2). The structure and density of the granules varied with each cell, but no K20-negative cells with accumulation of secretory granules in their basal cytoplasm could be observed. Therefore, it was confirmed that the K20-positive columnar cells were enteroendocrine cells and that all kinds of enteroendocrine cells in the duodenal epithelium expressed K20. In the enteroendocrine cells,



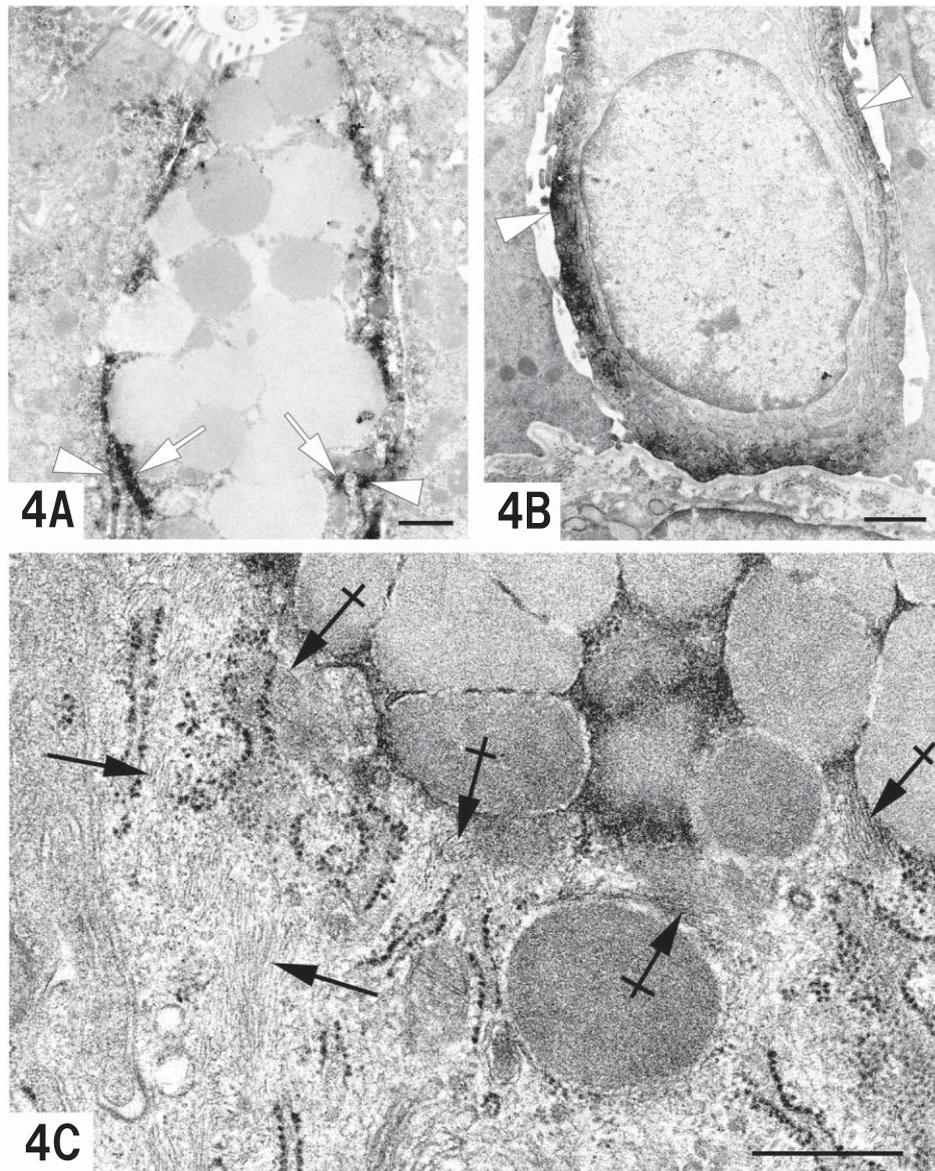
**Fig. 1.** Immunohistochemical staining of keratin 20 (K20) in the duodenum. Counterstained with hematoxylin. **A:** Goblet cells and a few special columnar cells in the epithelium and serous and mucous cells of the duodenal glands (*DG*) are selectively stained. Bar=50  $\mu$ m. **B, C:** Strong staining is observed in a few special columnar cells scattered throughout the epithelia of the villi (**B**) and crypt (**C**). A goblet cell (*G*) in the crypt is also faintly stained. Bar=5  $\mu$ m. **D:** A goblet cell in the epithelium covering villi is intensely stained. Bar=5  $\mu$ m. **E:** Mucous cells (*M*) and serous cells (*S*) are stained. Particularly, the edge of both cells are strongly stained. Bar=5  $\mu$ m.



**Fig. 2.** Immunoelectron microscopical staining of K20. **A, B:** Secretory granules are concentrated in the basal cytoplasm of the K20-positive columnar cells scattered throughout the epithelia of the villi (**A**) and crypt (**B**). In both cells, K20-containing filaments (white arrowheads) are detected in the perinuclear region and between this region and the apical cell membrane. Bar=2  $\mu$ m.



**Fig. 3.** Ultrastructure of an enteroendocrine cell in the villus epithelium. **A:** Low-magnification. Rectangles B and C indicate the site corresponding to Figure 3B and Figure 3C. Bar=3  $\mu$ m. **B:** Dense bundles of intermediate filaments (arrows), which radiate from the perinuclear region to the apical cell membrane, are observed in the apical cytoplasm. Bar=0.2  $\mu$ m. **C:** Intermediate filaments (arrows) are also concentrated around the nucleus. Bar=0.2  $\mu$ m.



**Fig. 4.** Goblet cells in the villus epithelium. **A, B:** Immunoelectron microscopical staining of K20 in a goblet cell. K20-containing filaments are detected just beneath the cell membrane (white arrowheads) and around the mass of mucigen granules (white arrows) in the upper (**A**) and basal (**B**) portions of a goblet cell in the villus epithelium. Bar=1  $\mu\text{m}$ . **C:** Ultrastructure of a goblet cell. Dense bundles of intermediate filaments are observed in the marginal region of the cell (arrows) and around the mass of mucigen granules (barred arrows). Bar=0.5  $\mu\text{m}$ .

K20-containing filaments were localized around the nucleus and formed a juxtannuclear network. They were also distributed from this network to the apical cell membrane. A quite similar distribution pattern of intermediate filaments was recognized ultrastructurally. As shown in Figure 3, intermediate filaments were concentrated in the perinuclear region and radiated from that region to the apical cell membrane.

#### **Localization of K20 in exocrine cells**

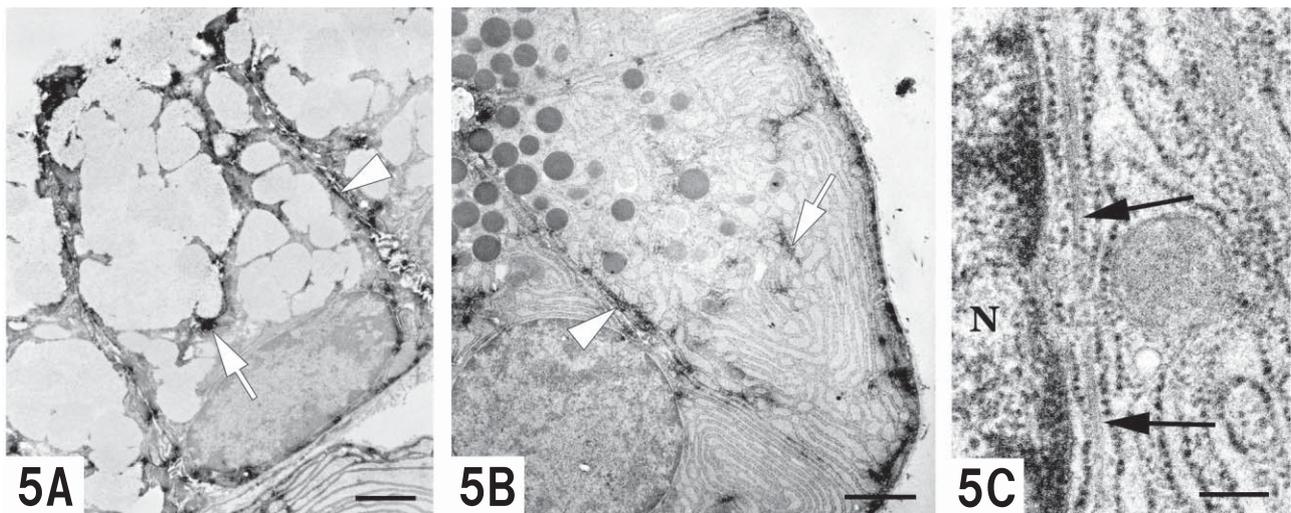
##### **a) Goblet cells**

A small amount of K20 could be detected in immature goblet cells in the crypt (Fig. 1C). After the goblet cells

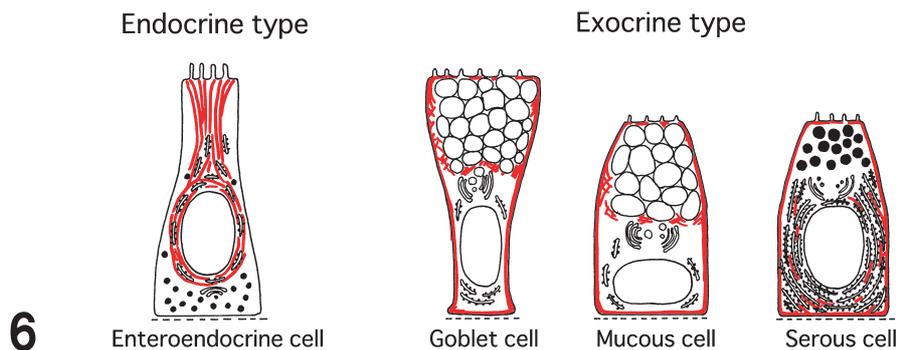
migrated to the villus base, the amount of K20 increased (Fig. 1D). As shown in Figure 4, K20-containing filaments were distributed just beneath the cell membrane and formed a thin peripheral network. They were also detected around the mass of mucigen granules. These distribution patterns were confirmed ultrastructurally. Dense bundles of intermediate filaments could be observed in the marginal region of the cells and around the mass of mucigen granules.

##### **b) Mucous cells of the duodenal glands**

Mucous cells of the duodenal glands of Brunner intensely expressed K20 (Fig. 1E). K20-containing filaments were distributed just beneath the cell membrane and around



**Fig. 5.** Mucous and serous cells of the duodenal gland. **A:** Immunoelectron microscopical staining of K20 in mucous cells. K20-containing filaments are detected just beneath the cell membrane (white arrowhead) and around the mass of mucigen granules (white arrow). Bar=2  $\mu$ m. **B:** Immunoelectron microscopical staining of K20 in serous cells. K20 is detected just beneath the cell membrane (white arrowhead) and among the cisternae of rough-surfaced endoplasmic reticulum (white arrow). Bar=2  $\mu$ m. **C:** Ultrastructure of a serous cell. Bundles of intermediate filaments (arrows) between the nuclear envelope and cisternae of rough-surfaced endoplasmic reticulum are clearly observed. Bar=0.2  $\mu$ m.



**Fig. 6.** Schematic illustration showing the localization of K20 in four kinds of secretory cells of the rabbit duodenum. Red lines indicate K20-containing intermediate filaments. In the endocrine cells, the filaments form a juxtannuclear network from which they extend to the apical cell membrane. In the three types of exocrine cells, the filaments form a peripheral network lying just beneath the cell membrane. In addition, the filaments also form a granule-associated network surrounding a mass of mucigen granules in the goblet cells and mucous cells, and distribute among the cisternae of the rough-surfaced endoplasmic reticulum in the serous cells.

the mass of mucigen granules (Fig. 5A), and the distribution pattern was quite similar to that in goblet cells. This distribution pattern was also confirmed ultrastructurally.

#### c) Serous cells of the duodenal glands

Serous cells of the glands also intensely expressed K20 (Fig. 1E). As shown in Figure 5B, K20-containing filaments were distributed just beneath the cell membrane similar to those of goblet cells and mucous cells. K20-containing filaments were also localized among the cisternae of rough-surfaced endoplasmic reticulum. The intermediate filament bundles among the cisternae were also recognized ultrastructurally (Fig. 5C).

The differences in localization of K20 in the four kinds

of secretory cells of the rabbit duodenum are schematically summarized in Figure 6.

## IV. Discussion

As shown in our results, among the 15 kinds of keratin detected in this study, K20 was selectively expressed in all secretory cells of the rabbit duodenum; namely, in enteroendocrine cells and goblet cells in the epithelium, and in mucous and serous cells in the duodenal glands. In contrast to the absorptive cells of the human duodenum, which predominantly express K20 [36], the immature and mature absorptive cells of the rabbit duodenum did not express K20. A small amount of this keratin was first recognized in these absorptive cells just before exfoliation [19]. This

discrepancy may be due to species difference. Therefore, it seems that K20 has a close relation to the secretory function in the rabbit duodenum.

K20 has been utilized as a valuable histochemical marker for many tumors [27, 29, 31, 35, 38] and metaplasia [4], although its biological role remains elusive. As shown in our results, localization of K20-containing filaments in the endocrine cells differed from that of three types of exocrine cells. In the endocrine cells, those filaments formed a juxtannuclear network from which they extended to the apical cell membrane. In the exocrine cells, on the other hand, those filaments formed a thin peripheral network lying just beneath the cell membrane. Recent evidence has suggested that intermediate filaments are associated with the intracellular signal transduction system [6, 12, 13, 18, 24, 26, 32, 40]. The possibility that K20-containing filaments in the enteroendocrine cells of the rabbit duodenum are also associated with that system should be considered for the following three reasons. First, these filaments were localized from the nuclear periphery to the apical cell membrane, which had many receptors for binding a specific extracellular signal. Second, keratin filaments have a high binding affinity to both the cell membrane [5, 47] and nuclear envelope [34, 48]. Third, keratin networks are dynamic structures and can be easily modified by the phosphorylation-dephosphorylation system [34, 40, 41]. K20 is expressed predominantly in undifferentiated epithelial cells at the early stage of organogenesis [2, 3, 37] and in some tumor cells [7, 23, 27, 35]. In these cells, K20-containing filaments form a cytoplasmic network or a thin peripheral network. These cells exhibit successive changes in cell shape for proliferation, movement or invasion. Therefore, it seems that K20-containing filaments have an especially dynamic character. A similar phenomenon has been noted in the superficial cells of uroepithelium, 'umbrella cells', which are subject to great changes owing to emptying and distension of urinary bladder. These cells also express a large amount of K20 as the most advantageous intermediate filament protein [43].

As shown in our results, in the case of the three types of exocrine cells, the K20-containing filaments formed a thin peripheral network lying just beneath the cell membrane. The shapes of these cells changed remarkably during the secretory cycle. Therefore, the exocrine cells of the duodenum may also select K20-containing filaments as an advantageous intermediate filament, since these filaments have a dynamic character, as mentioned above. In addition, K20-containing filaments formed a granule-associated network surrounding a mass of mucigen granules in the goblet cells of the epithelium and mucous cells of the duodenal glands. The shapes of the masses of mucigen granules also remarkably change during the secretory cycle. Therefore, K20-containing filaments seem to participate in the formation of this network in these two types of cells. On the other hand, the serous cells of the duodenal glands possessed K20-containing filaments among the cisternae of the endoplasmic reticulum in addition to the thin peripheral network. Intermediate filaments are involved in the storage and distribution of

organelles in the cytoplasm [16, 26, 39]. In the serous cells of the duodenal glands, K20-containing filaments may play a role in the distribution of the endoplasmic reticulum.

Keratin intermediate filaments consist of a specific combination of type I keratins and type II keratins [1, 14], and it has been supposed that the partner of K20 is K7 in carcinoma [7]. However, some recent studies have detected phenotypes of K7+/K20- and K7-/K20+ in some carcinomas [23, 29] and in pathological conditions of bile tract [4]. Zhou *et al.* [49], on the other hand, reported that K20 formed filaments with K8 in abnormal transgenic mice. However, a partner for K20 could not be identified in this study, since no keratin is co-localized with K20 in the secretory cells of the rabbit duodenum. The possibility that the filaments are composed of K20/K20 homodimers could not be ruled out, since Pang *et al.* [42] described the presence of a K13/K13 homodimer in the rabbit esophageal epithelium. However, this possibility could not be clarified in this study.

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